

# A Developmental Switch of Gene Expression in the Barley Seed Mediated by HvVP1 (Viviparous-1) and HvGAMYB Interactions<sup>1</sup>

Zamira Abraham, Raquel Iglesias-Fernández, Manuel Martínez, Ignacio Rubio-Somoza, Isabel Díaz, Pilar Carbonero, and Jesús Vicente-Carbajosa

The accumulation of storage compounds in the starchy endosperm of developing cereal seeds is highly regulated at the transcriptional level. These compounds, mainly starch and proteins, are hydrolyzed upon germination to allow seedling growth. The transcription factor HvGAMYB is a master activator both in the maturation phase of seed development and upon germination, acting in combination with other transcription factors. However, the precise mechanism controlling the switch from maturation to germination programs remains unclear. We report here the identification and molecular characterization of *Hordeum vulgare* VIVIPAROUS1 (HvVP1), orthologous to ABA-INSENSITIVE3 from *Arabidopsis thaliana*. HvVP1 transcripts accumulate in the endosperm and the embryo of developing seeds at early stages and in the embryo and aleurone of germinating seeds up to 24 h of imbibition. In transient expression assays, HvVP1 controls the activation of *Hor2* and *Amy6.4* promoters exerted by HvGAMYB. HvVP1 interacts with HvGAMYB in *Saccharomyces cerevisiae* and in the plant nuclei, hindering its interaction with other transcription factors involved in seed gene expression programs, like BPBF. Similarly, this interaction leads to a decrease in the DNA binding of HvGAMYB and the Barley Prolamine-Box binding Factor (BPBF) to their target sequences. Our results indicate that the *HvVP1* expression pattern controls the full *Hor2* expression activated by GAMYB and BPBF in the developing endosperm and the *Amy6.4* activation in postgerminative reserve mobilization mediated by GAMYB. All these data demonstrate the participation of HvVP1 in antagonistic gene expression programs and support its central role as a gene expression switch during seed maturation and germination.

Seed development after fertilization has been classically divided into two major phases: zygotic embryogenesis and maturation. The maturation phase is characterized by gene expression programs devoted to the synthesis and accumulation of reserve compounds, among them seed storage proteins (SSPs), and, later, to the acquisition of desiccation tolerance, with an important role for the late embryogenic abundant (LEA)

proteins (Vicente-Carbajosa and Carbonero, 2005). In most species of the Spermatophyta, seeds acquire a quiescent state at the end of the maturation phase that is resumed by the germination process, which is generally accepted to comprise the germination *sensu stricto* from seed imbibition to root emergence that is followed by reserve mobilization. These two distinct phases are independently regulated and influenced by genetic and environmental factors (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Nonogaki et al., 2007; Holdsworth et al., 2008; Iglesias-Fernández et al., 2011b; González-Calle et al., 2015). During early postgermination (reserve mobilization), several genes encoding hydrolytic enzymes (i.e.  $\alpha$ -amylases, proteases, and lipases) catalyze the hydrolysis of carbohydrates, proteins, and lipids present in the seed storage tissues, such as the endosperm of monocotyledonous species and the cotyledons of dicotyledonous ones, with the aim of nourishing the growing embryo before attaining its full photosynthetic capacity (Pritchard et al., 2002; González-Calle et al., 2014; Iglesias-Fernández et al., 2014).

The comprehensive molecular and genetic mechanisms controlling seed development and germination still remain elusive, due mainly to the many environmental and

intrinsic parameters influencing it. From a physiological point of view, it is generally accepted that an increased abscisic acid ABA-GA ratio determines the progression of seed maturation by promoting the expression of SSPs, LEAs, heat shock proteins, and anthocyanin biosynthesis genes (Reidt et al., 2000; Koornneef et al., 2002; Vicente-Carbajosa and Carbonero, 2005; Braybrook and Harada, 2008; Finkelstein et al., 2008). By contrast, an increased GA-ABA ratio is the most important factor for the integration of the environmental and intrinsic signals for the occurrence of germination. This enhanced GA-ABA balance raises the expression of hydrolase genes such as those encoding cell wall-remodeling enzymes, involved in the weakening of the embryo-surrounding tissues during germination *sensu stricto*, and of reserve mobilization enzymes post germination (Gubler et al., 1995, 1999; Mena et al., 2002; Iglesias-Fernández et al., 2011a, 2011c; González-Calle et al., 2015).

In cereal seeds, the transcription factor (TF) of the B3 family VIVIPAROUS1 (VP1), orthologous to ABA-INSENSITIVE3 (ABI3) from *Arabidopsis* (*Arabidopsis thaliana*), is a key component of ABA signaling and is required for the expression of the maturation program (McCarty et al., 1991; Parcy et al., 1994; Jones et al., 1997; Nambara et al., 2000; Suzuki et al., 2001; Lara et al., 2003; Zeng et al., 2003; To et al., 2006; Swaminathan et al., 2008; Yan et al., 2014). The VP1 mutants, like *vp1* in maize (*Zea mays*) and *abi3* in *Arabidopsis*, are characterized by the incapacity of entering the quiescent state at the end of the maturation phase and by displaying premature features of the subsequent germination and postgermination phases, leading to a precocious germination of the seeds still in the mother plant (preharvest sprouting; McCarty et al., 1989; Gubler et al., 2005). It has been described previously that the VP1 TF, through its binding to the specific RY cis-element (5'-CATGCA-3') and through protein-protein interactions with other TFs of the bZIP family, regulates the expression of seed maturation-specific genes, such as those encoding the Em protein in the wheat (*Triticum aestivum*) caryopsis and the C1 (MYB-like) TF, a key regulator of the anthocyanin biosynthesis pathway in maize (Paz-Ares et al., 1986; Hattori et al., 1992; Vasil et al., 1995). VP1 also has been associated with the repression of postgermination genes, since in the maize *vp1* mutant,  $\alpha$ -amylase activity is precociously observed in the kernels while still in the cob, indicating that all necessary factors for its activation are present already during the maturation phase and in support of the idea that the VP1 protein must be preventing this hydrolytic activity (Hoecker et al., 1999; Rodríguez et al., 2015).

In the last 20 years, an important number of barley (*Hordeum vulgare*) TFs, such as GAMYB, BPBF (HvDOF24), SAD (HvDOF23), and BLZ2 (a bZIP orthologous to the maize OPAQUE2), have been characterized as central regulators of gene expression in the seed maturation and postgermination (reserve mobilization) programs (Gubler et al., 1995; Mena et al., 1998, 2002; Oñate et al., 1999; Diaz et al., 2002; Isabel-LaMoneda et al., 2003). However, their possible relationship with barley

VP1 has not been investigated, although similar interactions have been reported in other cereal species (Hill et al., 1996; Hobo et al., 1999) and for ABI3 and AtbZIP10/AtbZIP25 (BLZ2 orthologs) with a role in activating the expression of SSP genes upon seed maturation in *Arabidopsis* (Lara et al., 2003).

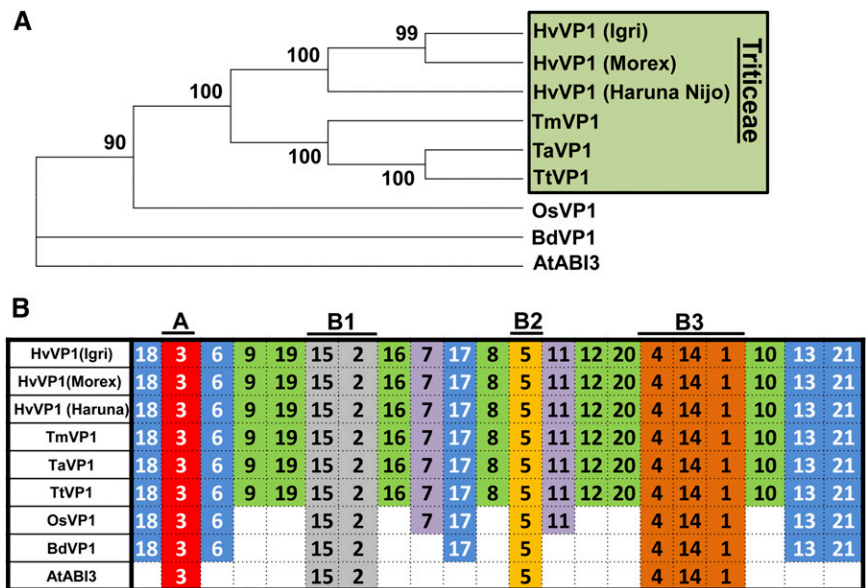
In this work, the barley VP1 gene (*HvVP1*) has been characterized and its expression localized, by mRNA in situ hybridization experiments, to the embryo and the endosperm of barley seeds, both during maturation and upon germination. In transient expression experiments in immature barley endosperms and in aleurone layers of germinating seeds, HvVP1 interferes with the transcriptional activation exerted by GAMYB on the promoters of the genes *Hor2* and *Amy6.4*, encoding a B-hordein (SSP) and a high-pI  $\alpha$ -amylase, respectively. By yeast two-hybrid (Y2H) and bimolecular fluorescence complementation assays, the HvVP1-GAMYB protein interaction was confirmed in vivo and in plant nuclei. Interestingly, the presence of HvVP1 not only diminishes the GAMYB-BPBF protein interaction in yeast three-hybrid (Y3H) assays, but HvVP1 also decreases the binding affinities of GAMYB and BPBF for their corresponding cis-elements in the promoters of the *Hor2* and *Amy6.4* genes in electrophoretic mobility shift assays (EMSAs). All these data indicate a central role for HvVP1 as a gene expression switch at key stages of seed maturation and germination.

## RESULTS

### *HvVP1* Sequence Identification and Phylogenetic Dendrogram

The sequence and structure of the predicted gene *HvVP1*, isolated from a barley 'Igri' genomic library (Supplemental Fig. S1), was confirmed by sequencing the corresponding open reading frame (ORF) derived from a PCR-amplified complementary DNA (cDNA) from developing barley seeds (20 d after pollination [dap]). This sequence was compared with other VP1 sequences from different barley cultivars (cv Igri, cv Morex, and cv Haruna Nijo), with its orthologs in other Gramineae species deposited in public databases, such as wheat (*TaVP1*), *Triticum turgidum* (*TtVP1*), *Triticum monococcum* (*TmVP1*), *Brachypodium distachyon* (*BdVP1*), and rice (*Oryza sativa*; *OsVP1*), and with *AtABI3* from *Arabidopsis*. The *HvVP1* gene is a single-copy gene (Supplemental Fig. S2). The deduced HvVP1 protein sequence from cv Igri is more than 99% identical with those from cv Morex (Mayer et al., 2012) and cv Haruna Nijo (Matsumoto et al., 2011) and with the VP1 partial sequence of cv Himalaya in GenBank (AAO06117.1; Casaretto and Ho, 2003; Supplemental Fig. S3). A phylogenetic tree including these sequences has been constructed (Fig. 1A), and HvVP1 is included on the same branch as those of the Triticeae tribe species, with a bootstrap value of 100%. This phylogenetic tree is

**Figure 1.** A, Phylogenetic tree with the deduced amino acid sequences of the VP1 genes from distinct barley cultivars (cv Igri, cv Morex, and cv Haruna Nijo), with wheat (TaVP1), *T. turgidum* (TtVP1), *T. monococcum* (TmVP1), *B. distachyon* (BdVP1), and rice (OsVP1), and with AtABI3 from Arabidopsis. Bootstrapping values are specified at the branches. B, Distribution of the conserved motifs among the deduced protein sequences in the dendrogram (A), found by means of MEME analysis. A, B1, B2, and B3 correspond to conserved domains, as described by Nakamura and Toyama (2001) and Marella and Quatrano (2007).



further supported by the occurrence of common motifs (MEME analysis; Fig. 1B; Table I). All sequences share motifs 1, 2, 3, 4, 5, 14, and 15, with the exception of motif 3, which is only partially conserved in AtABI3. According to Nakamura and Toyama (2001), motif 3 corresponds to the activation domain (A), motifs 15 and 2 form the B1 domain, motif 5 matches with the B2 domain, and motifs 1, 4, and 14 integrate the B3 (DNA-binding) domain. VP1 proteins from members of the Triticeae tribe (TaVP1, TtVP1, TmVP1, and HvVP1) share motifs 8, 9, 10, 12, 16, 19, and 20, and all the VP1

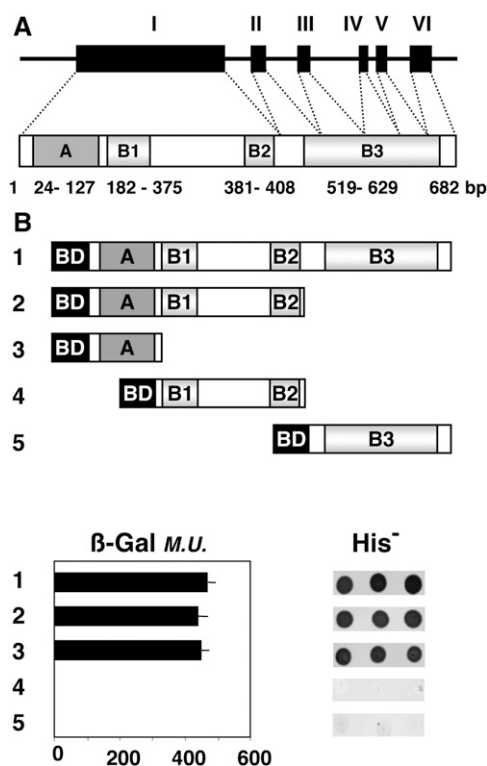
proteins in the Poaceae genomes compared share motifs 6, 13, 17, 18, and 21.

The deduced amino acid sequences encoded by these VP1 genes have nuclear localization signals (RKRR), molecular masses of 72 to 75 kD, and pI between 6.4 and 8.8 (Supplemental Table S1). The HvVP1 gene contains six exons (Fig. 2A, solid bars) and five introns (Fig. 2A, lines) as determined by comparing the genomic DNA and cDNA clones. While domains A, B1, and B2 (containing the nuclear localization signal RKRR; Graeber et al., 2010) are encoded in the first

**Table I.** Sequences of conserved amino acid motifs (MEME; Bailey et al., 2009) of the VP1 orthologous genes from barley, *T. monococcum*, *T. turgidum*, wheat, rice, *B. distachyon*, and Arabidopsis

The nuclear localization signal RKRR is underlined in boldface.

Motif	E	Consensus Sequence
1	4.0e-377	DIGTS[QR]VW[SN]MRYRFPNPNKSRMYLLENTGDFVRSNELQEGDFIV[LI]YSDVK
2	8.8e-310	NNR[DE]CISAEDLRSL[RK]RSTIEAAARLGGGRQGTMLLKLITWVQNH
3	3.3e-175	DDFMFA[QD]DTFPALPDFCLSSPSS[TN]FSSSSSSNSSAF
4	1.1e-150	DKNLRFLQKVLKQSDVG[TS]LGRIVLPK[KE][EA]
5	3.6e-109	MEP[AS]AT[ARK]EARKRRMARQRRLS[CS]
6	1.6e-097	EPSEPAAGDG[MV]DDL[SA]DID[HQ]LLDFAS[IL][NS]
7	1.1e-149	YFEP[TA]ETGAAATSWMPYQAFSPT[AG]SYGGEE[M]YFQQGCST
8	1.4e-151	DMHAGAWPLQYAAFPAGATSAGTQTPMPPPG[AP]VPQFPAAPGFAGQFPQ
9	5.4e-101	DDVPWDDEPLFPDVGMMLEDVISEQQ[QL]QQ
10	5.7e-101	KYLIRGVKVR[AAQ]Q[EG]LAKHKN[AG]SPEKGGAS[DE]VKA
11	2.1e-112	LQQQRSQQLNLSQIQTGFGPQE[PQ]SPRAAHSAPV
12	1.5e-123	[HG]W[SG][PG][PL][AW][VT]Q[IAQ][QA][PV][HQ]GQLM[IV]QVPNPLSTKSNSSRQKQKQPSDAAARPPSGGA
13	5.3e-087	EDGGCKEKSHPGVRRSRQEA[AS]
14	5.0e-080	ETHLPELKT[GR]DGSI
15	2.4e-071	GGG[GS][STG]GSAADDLP[RAL]FFMEWL[TK]
16	8.2e-074	LQKKRPRVGAMDQEAPPAGGQLPSPGANP
17	4.4e-042	SSV[VA]VSSQPFSPAA
18	8.1e-042	[AH]P[QR][GR][GS][GA]P[HR]RGK[GA]PAVEIR[HQ]GE
19	1.2e-036	[HY]G[AT][AG]GR[TV]AS[DH]AA[AG]GGGEDAFM
20	2.6e-029	[ST][PQ][QH]R[PQ]GQA[AS]AS[DN]KQRQQGA[RS][TR][PT]AAAP[AP]AG
21	1.6e-026	MNQMAVSI



**Figure 2.** A, *HvVP1* transcript splicing model. Exons I to VI are indicated as black bars. Motifs A, B1, B2, and B3 are as in Figure 1. B, Functional assay in yeast for the activation capacity of the *HvVP1* protein. Different constructs (1–5) were generated as fusions to the GAL4 DNA-binding domain (black box at the N terminus), and the activation capacity was assayed in two alternative reporter systems:  $\beta$ -Galactosidase ( $\beta$ -Gal) activity (*LacZ* reporter gene; Miller's units [M.U.] in liquid medium) and *HIS3* (growth capacity in His-depleted [His<sup>−</sup>] medium).

exon, the B3 DNA-binding domain is translated from exons II to VI.

#### Activation Properties of the *HvVP1* Protein

To investigate the activation capacity of *HvVP1* in yeast (*Saccharomyces cerevisiae*), a series of constructs containing different regions of the *HvVP1* ORF was prepared (Fig. 2B). These regions were cloned as translational fusions to the GAL4 DNA-binding domain into a yeast expression plasmid and tested as effectors for their capacity to transactivate two alternative reporter genes: *LacZ*, encoding a  $\beta$ -Gal assayed in liquid cultured cells; and *HIS3*, which promotes yeast growth in a His-depleted agar medium. Both in qualitative (His depleted) and quantitative ( $\beta$ -Gal; M.U.) assays, *HvVP1* acts as a potent activator in yeast (approximately 450  $\beta$ -Gal M.U.). However, when domain A is deleted, the reporter expression disappears, indicating that this region is essential for *HvVP1* being a transcriptional activator in yeast.

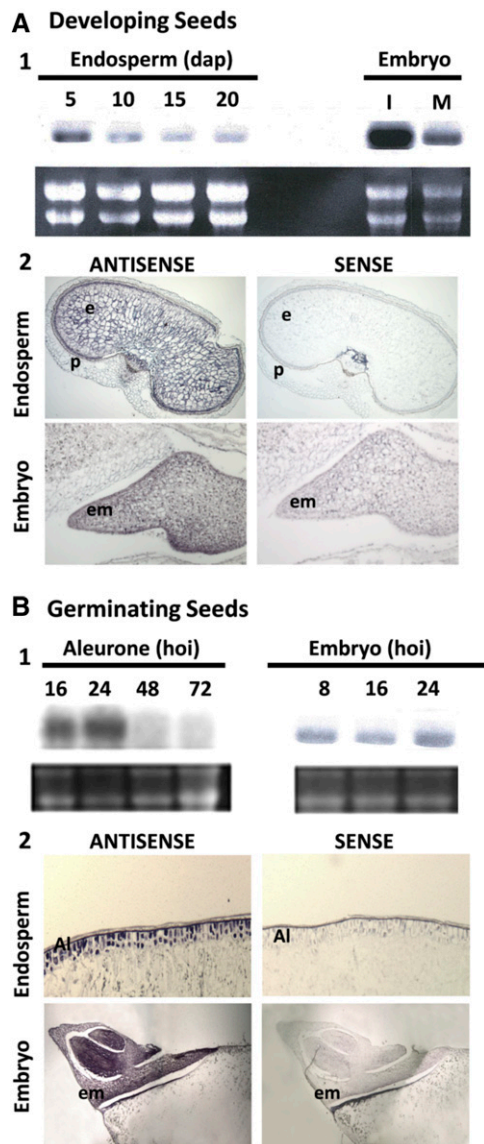
#### *HvVP1* Is Expressed in the Embryo and in the Endosperm during Seed Maturation and Germination

Cereal VP1 proteins, such as Arabidopsis ABI3, belong to a subfamily of B3 TFs controlling seed gene expression (Swaminathan et al., 2008). Consequently, it is expected that the *HvVP1* mRNA should be expressed during barley seed maturation and germination. To test this, total RNA was isolated from developing deembryonated seeds (endosperms at 5, 10, 15, and 20 dap), immature (20 dap) and mature dry embryos, and aleurones and embryos of germinating seeds at different times of imbibition (8, 16, 24, 48, and 72 h of imbibition [hoi]). Northern-blot analysis (Fig. 3) shows that the *HvVP1* transcripts are found both in developing endosperms, attaining maximum levels at 5 dap and decreasing thereafter (20 dap), and in immature and mature embryos, being more abundant at the immature stage (Fig. 3A). The *HvVP1* transcripts are expressed upon seed germination in the aleurone layer, reaching a peak at 24 hoi, and also in embryos (Fig. 3B). mRNA in situ hybridization experiments during seed maturation (20 dap) and upon germination (24 hoi) reveal that *HvVP1* transcripts are present throughout the seed (embryo and endosperm) during maturation, while its expression is restricted to the embryo and the aleurone layer upon germination (Fig. 3). The *HvVP1* transcripts are not found in leaves and roots of adult barley plants (data not shown).

#### *HvVP1* Counteracts the *HvGAMYB* Transactivation Exerted on Endosperm-Specific Genes during Seed Maturation and Germination

*HvGAMYB* is a barley MYB-R2R3 protein that is a transcriptional activator of seed storage protein genes (B-hordeins: *HvHor2*) during seed maturation and of hydrolase genes ( $\alpha$ -amylases: *HvAmy6.4*) needed for reserve mobilization post germination (Gubler et al., 1995, 1999; Diaz et al., 2002).

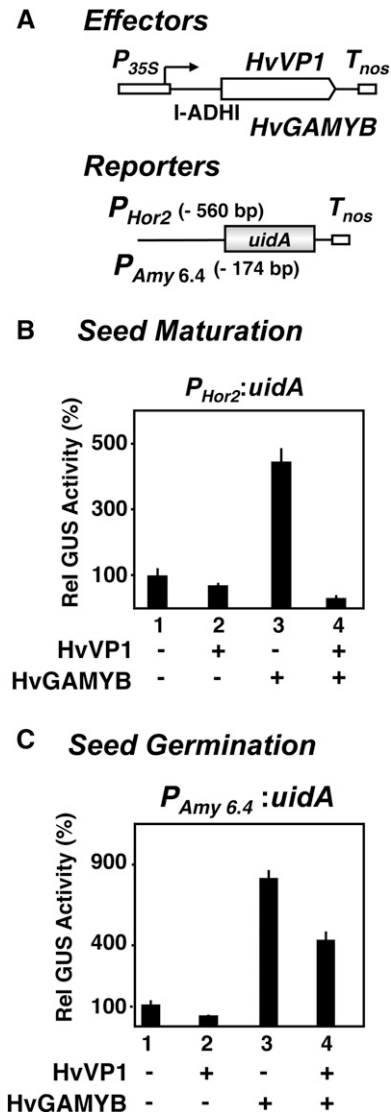
Taking into account that both *HvVP1* (Fig. 3) and *HvGAMYB* share similar spatial expression patterns in the seed, as occurs with other important seed-specific TFs (Supplemental Fig. S4), transient expression assays were done by cobombardment in barley developing endosperms and in germinating aleurones to investigate their mutual effects. In Figure 4A, a schematic description of the constructs used in the transactivation study is shown. The TFs *HvVP1* and *HvGAMYB* were used as effectors. As reporter constructs, the *HvHor2* promoter (−560 bp upstream of the ATG initiation codon) and the *HvAmy6.4* gene promoter (−174 bp) were fused to the GUS reporter gene (*P<sub>Hor2</sub>::uidA* and *P<sub>Amy6.4</sub>::uidA*). As shown in Figure 4B, while cobombardment of developing barley endosperms with *HvVP1* and *P<sub>Hor2</sub>::uidA* reduces GUS activity to half that driven by the *P<sub>Hor2</sub>::uidA* construct alone, cotransfection with *HvGAMYB* and *P<sub>Hor2</sub>::uidA* provokes an increment of GUS activity of approximately 5-fold. When both TFs (*HvVP1* and *HvGAMYB*) are cobombarded together with *P<sub>Hor2</sub>::uidA*, the positive transactivation of *P<sub>Hor2</sub>::uidA*



**Figure 3.** *HvVP1* expression analyses by northern blot and mRNA in situ hybridization analyses in barley developing (A) and germinating (B) seeds. A.1, Northern-blot analysis. Eight micrograms of total RNA from developing endosperms (from 5, 10, 15, and 20 dap) and immature (I; 25 dap) and mature (M) embryos was loaded in each lane and hybridized to an *HvVP1* gene-specific probe. A.2, *HvVP1* transcript localization in longitudinal sections of developing (25 dap) seeds by mRNA in situ hybridization. B.1, Northern-blot analysis in germinating aleurone (16, 24, 48, and 72 hoi) and germinating embryo (8, 16, and 24 hoi). B.2, *HvVP1* transcript localization in germinating barley seeds (36 hoi) by mRNA in situ hybridization assay. Left and right images correspond to hybridizations with *HvVP1* antisense and sense probes, respectively. The ethidium bromide-stained ribosomal RNA images are included as loading controls. e, Endosperm; em, embryo; p, pericarp; s, scutellum.

exerted by *HvGAMYB* diminishes drastically (Fig. 4B). Similarly, Figure 4C shows that while cobombardment of *HvVP1* with *P<sub>Amy6.4</sub>::uidA* diminishes GUS activity to half that driven by the reporter gene alone (*P<sub>Amy6.4</sub>::uidA*), cotransfection of *HvGAMYB* and *P<sub>Amy6.4</sub>::uidA* increases

GUS activity more than approximately 8-fold, and cobombardment of both TFs reduces the GUS activity of the reporter construct to half that obtained when *HvGAMYB* is the only transfected TF. This repressor effect of *HvVP1* is independent of the presence of GA (1  $\mu$ M) added to the incubation medium (Supplemental Fig. S5). In summary, *HvVP1* is a transcriptional repressor of maturation and germination seed genes.

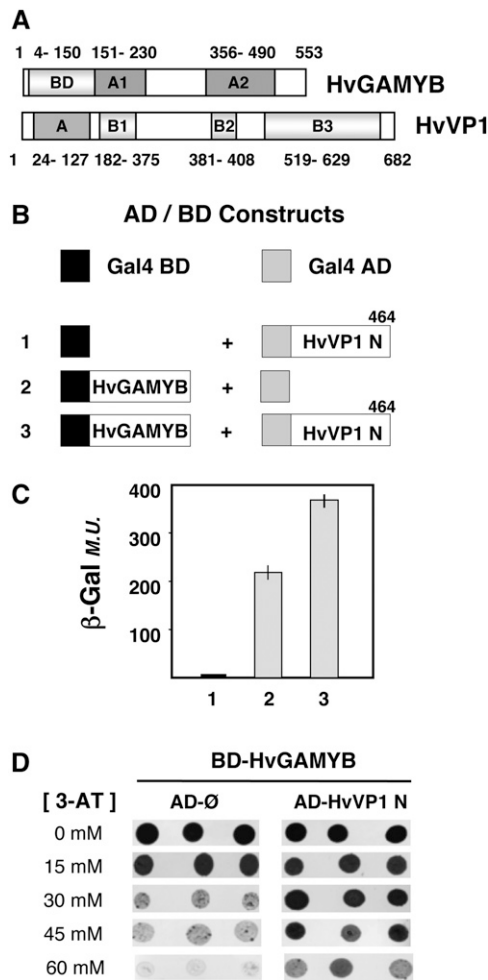


**Figure 4.** Transactivation assays using as effectors the TFs *HvVP1* and *HvGAMYB*. The *Hor2* and *Amy6.4* gene promoters driving the expression of the *uidA* gene (GUS activity) were used as reporters. A, Schematic representation of the effector and reporter constructs used in the analyses. B, Cobombardment in barley developing endosperms of the effector and reporter combinations indicated. C, The effector and reporter constructs designated were cobombarded in germinating aleurones from barley kernels. The relative amounts of reporter and effector plasmids used in these assays correspond to a 1:1 ratio. Values are means  $\pm$  SE of three independent replicates.

## HvVP1 Interacts with HvGAMYB in the Y2H System and in Plant Nuclei

To explore whether the repression exerted by the HvVP1 TF on the transactivation mediated by HvGAMYB on seed-specific genes during maturation and germination could be due to a direct HvVP1-HvGAMYB protein-protein interaction, Y2H and bimolecular fluorescence complementation assays were done.

*LacZ* and *HIS3* reporter activities were measured in Y2H assays (Fig. 5). The HvVP1 N-terminal cDNA (N), encoding 464 amino acid residues, spanning domains A, B1, and B2 (Fig. 2A), was translationally fused to the yeast *GAL4* activation domain (AD), and the full-length



**Figure 5.** Protein-protein interaction by Y2H assays. A, The structures of HvGAMYB and HvVP1 are depicted, showing the locations of functionally relevant domains within the proteins. B, HvGAMYB (complete ORF) or HvVP1 N<sup>464</sup> (the 464 N-terminal amino acid residues) expressed as translational fusions to the GAL4 DNA-binding domain (BD) and activation domain (AD), respectively. C and D, Interaction capacity assayed by measuring the  $\beta$ -Gal activity (*LacZ* reporter gene; M.U. in liquid medium; C) and by evaluating the yeast growth capacity in a His-depleted medium (reporter gene *HIS3*) with increasing concentrations of 3-aminotriazole (3-AT; D).

cDNA of HvGAMYB was fused to the *GAL4* binding domain (BD; Fig. 5B). In Figure 5C, yeast cells (strain SFY526) transformed with the *GAL4BD-HvGAMYB* construct show detectable background levels of *LacZ* reporter activity, and this activity increases sharply (approximately 400  $\beta$ -Gal M.U.) when this strain is cotransformed with the *GAL4AD-HvVP1* construct. Expression of the *HIS3* gene (strain HF7C), conferring His auxotrophy, was used to confirm the interaction between HvGAMYB and HvVP1N (Fig. 5C). Yeast cells containing *GAL4BD-HvGAMYB* and the *GAL4AD-Ø* constructs are not able to grow in 3-aminotriazole concentrations higher than 30 mM. However, when transformed with *GAL4BD-HvGAMYB* and *GAL4AD-HvVP1N* constructs, yeast cells grow even at 60 mM 3-aminotriazole. All these results support the idea that the HvGAMYB and HvVP1 proteins interact in vivo.

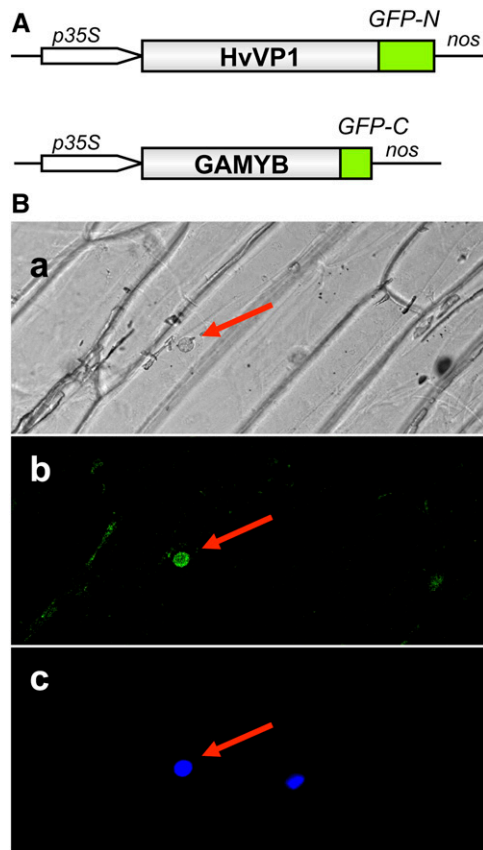
In order to corroborate the HvVP1-HvGAMYB interaction, bimolecular fluorescence complementation experiments were done in planta. With this aim, HvVP1 and HvGAMYB ORFs were translationally fused to the N- and C-terminal fragments, respectively, of the GFP-encoding gene (Diaz et al., 2005) and used for cobombardment experiments in onion (*Allium cepa*) epidermal cells. Microscopic observations show that the GFP fluorescence is reconstituted and targeted to the nucleus, indicating that HvVP1 and HvGAMYB proteins interact in plant nuclei (Fig. 6). As expected, there is no detectable GFP when the GFP fragments are bombarded alone (data not shown).

## HvVP1 Interferes with the HvGAMYB-BPBF Interaction in Vivo and with Their DNA Binding in EMSA

Since we showed that HvVP1 and GAMYB proteins can interact physically, we wanted to determine whether the HvVP1 protein could interfere with the binary complex formed by HvGAMYB and BPBF-HvDOF24, as reported previously (Diaz et al., 2002). For this purpose, a Y3H system was used, based on the pBridge vector, which allows the simultaneous expression of two proteins: in this case, the GAL4BD-BPBF translational fusion and the HvVP1 ORF, the latter conditionally expressed under the control of the *Met-25* promoter (*P<sub>Met-25</sub>*) that responds to the Met supply in the medium. The *GAL4AD-HvGAMYB* construct was used as the third component of the system (Fig. 7A). In this Y3H system and in the absence of Met in the yeast growth medium, the joint expression of HvGAMYB-AD and BPBF-BD increases the reporter gene activity (greater than 80  $\beta$ -Gal M.U.), compared with the expression of BPBF alone (approximately 40  $\beta$ -Gal M.U.), as expected for a positive interaction between the two proteins. However, this activity diminishes when HvVP1 is present (approximately 60  $\beta$ -Gal M.U.; Fig. 7B), suggesting that HvVP1 interferes with the interaction between HvGAMYB and BPBF.

The specific DNA-protein interactions between BPBF (HvDOF24) and its corresponding cis-motif (prolamine

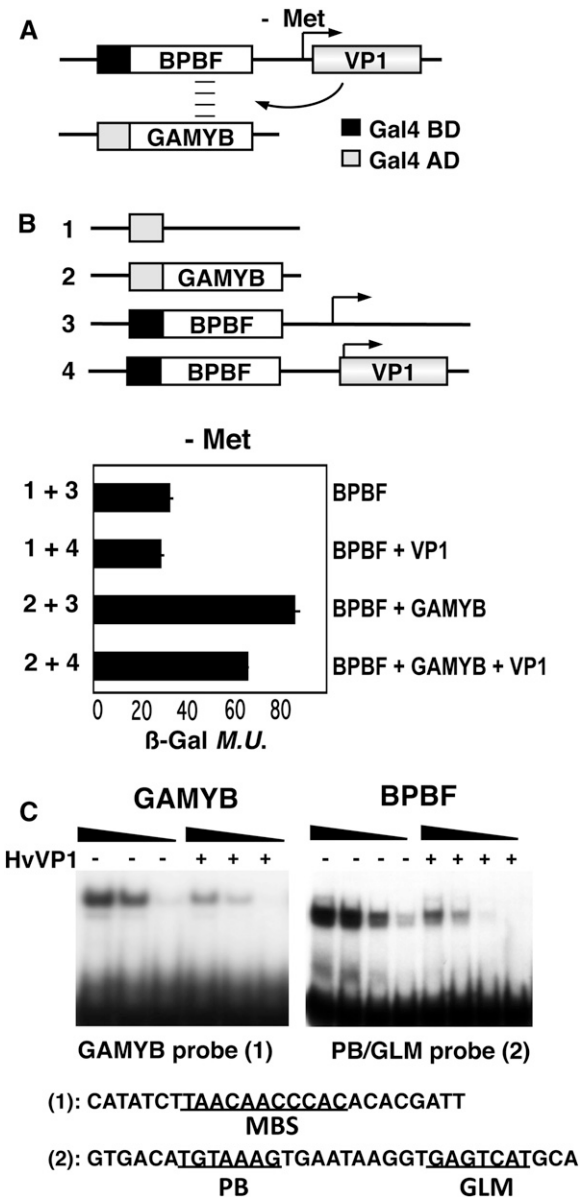




**Figure 6.** In planta interaction of HvVP1 and HvGAMYB. A, Structures of gene constructs used in bimolecular fluorescence complementation assays using particle gun transformation of onion epidermal cells. B, GFP reconstruction in the nuclei of cells transformed with a combination of the constructs described above (b). Bright-field images (a) and 4',6-diamidino-2-phenylindole staining (c) was used to reveal nuclei within the cells.

box [PB]; 5'-TGTAAG-3') and between HvGAMYB and the MYB-R2R3 box (5'-TAACAAC-3') at the promoter region of the *HvHor2* gene have been reported previously (Mena et al., 1998; Diaz et al., 2002). The potential effect of HvVP1 in modifying these protein-DNA interactions was investigated by EMSA (Fig. 7C). HvVP1, HvGAMYB, and BPBF proteins produced in *Escherichia coli* were assayed for their binding activities using two <sup>32</sup>P-labeled DNA probes containing the MYB-R2R3 and the PB-binding sites, derived from the *HvHor2* promoter (Fig. 7C). While a clear retarded band is observed when HvGAMYB or BPBF protein extracts are incubated with their respective probes, the addition of increasing amounts of HvVP1 protein to the reactions leads to fainter, or even to the absence of, retarded bands. Notably, HvVP1 does not bind to any of the probes used containing the PB and GLM motifs (target-binding sites of BPBF and BLZ2, respectively) or the MYB-R2R3 box (target-binding site of GAMYB), in support of its direct interference with the binding of these proteins. Interestingly, the modification of DNA

binding by HvVP1 seems to be specific, since the intensity of the retarded bands in EMSAs for SAD (HvDOF23) was decreased in the presence of HvVP1,



**Figure 7.** Protein-protein interactions of HvVP1, GAMYB, and BPBF in Y3H experiments and the effect of HvVP1 on GAMYB and BPBF DNA-binding capacities. A, Schematic representation of the constructs used in the Y3H assays, where the BPBF ORF was translationally fused to the GAL4 DNA-binding domain (BD) in the pBridge plasmid harboring HvVP1 under the control of a Met-repressible promoter. The GAMYB ORF was translationally fused to the GAL4 activation domain (AD) in pGAD424. B, Schematic representation of the constructs used for the Y3H assays and quantification of  $\beta$ -Gal activity (expressed as M.U.) in liquid assays using the constructs represented above. The proteins expressed in Met-depleted medium are indicated at right. C, EMSAs of HvGAMYB and BPBF proteins in the absence (-) or presence (+) of the HvVP1 protein. Incubations were done with a specific <sup>32</sup>P-labeled probe containing a target site for the corresponding TFs: GLM (G-box-like motif), BLZ2; PB, BPBF; MBS (MYB-binding site), GAMYB.

while the contrary effect (enhancement) was observed for the bZIP protein BLZ2 (Supplemental Fig. S6)

### HvVP1 Counteracts the Transcriptional Activation of BPBF on the *HvHor2* Gene Promoter

BPBF is a barley DOF TF that acts as a transcriptional activator of the endosperm-specific gene *HvHor2* during seed maturation (Mena et al., 1998), and the BPBF transcripts are abundant during the seed maturation and germination phases (Supplemental Fig. S4).

As BPBF shares similar spatial expression patterns during seed development with *HvVP1* and the binding of its encoded protein for the PB box is decreased drastically by *HvVP1* (EMSA in Fig. 7C), we decided to perform transient expression assays by particle bombardment in developing barley endosperms to explore the in planta effects of the coexpression of both TFs. Figure 8A schematically shows the constructs used in the transactivation study, where BPBF and *HvVP1* were tested as effectors of GUS reporter activity driven by the  $P_{Hor2}:uidA$  construct. As shown in Figure 8B, cobombardment of barley developing endosperms with the *HvVP1* effector and the  $P_{Hor2}:uidA$  reporter diminishes GUS activity to half that of  $P_{Hor2}:uidA$  alone, whereas cotransfection with BPBF and  $P_{Hor2}:uidA$  provokes an increment of GUS activity of

approximately 7-fold. When both *HvVP1* and BPBF are cobombarded, the positive  $P_{Hor2}:uidA$  transactivation exerted by BPBF is reduced to approximately one-half. This result demonstrates that *HvVP1* counteracts the activation of BPBF upon the *HvHor2* gene in developing barley endosperms.

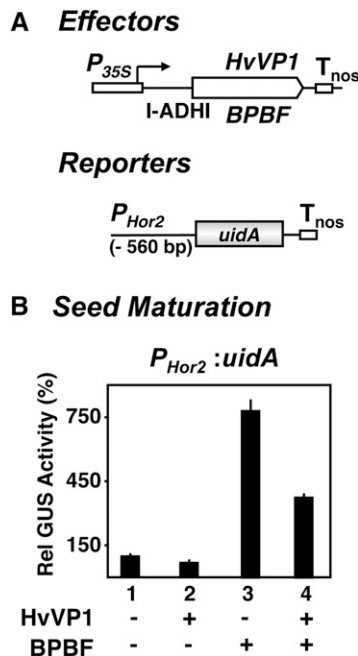
## DISCUSSION

In this work, we have identified the *HvVP1* gene of cv Igri and explored its function in the control of gene expression programs during seed development and germination. In particular, we address the role of *HvVP1* through its interaction with *HvGAMYB*, a key TF that participates in the regulation of both maturation and germination genes in the barley seed. Our work unveils important features of *HvVP1* action derived from its precise spatiotemporal expression patterns in the seed and its capacity to modulate regulatory complexes in which *HvGAMYB* participates. We propose a general model (Fig. 9) that integrates the data presented here with current knowledge derived from previous studies in the cereal seed.

### HvVP1 Effects during Barley Seed Development

In barley, several TFs such as *HvGAMYB*, BPBF, and the bZIPs BLZ1 and BLZ2 (Vicente-Carbajosa et al., 1997, 1998; Mena et al., 1998; Oñate et al., 1999; Diaz et al., 2002), acting as part of regulatory complexes, have been reported to participate in the regulation of maturation genes like SSPs (B-hordein; gene *HvHor2*). Among them, the *HvGAMYB* protein activates the transcription of the *HvHor2* gene by binding to the corresponding MYB-R2R3 box (5'-TAACAAC-3') in its promoter. Moreover, *HvGAMYB* interacts physically with BPBF (Diaz et al., 2002), and together, they significantly increase the transactivation of the *HvHor2* promoter in developing barley endosperms as compared with the transactivation of each TF considered separately.

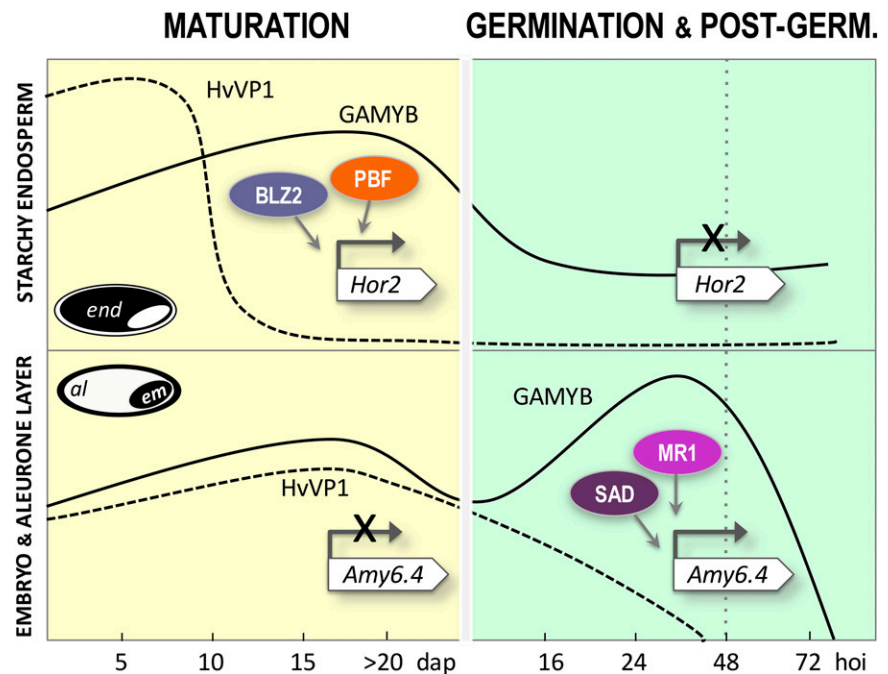
In this work, we have performed a detailed expression analysis of *HvVP1* during seed development to define its precise expression pattern. Since *HvVP1* is a known regulator of seed maturation genes, we wanted to survey its potential effect on key regulatory factors controlling this process. As a first step, we explored *HvVP1* action on the transactivation of the *HvHor2* promoter mediated by *HvGAMYB* and demonstrate that *HvVP1* interferes with its activation capacity (Fig. 4B). Furthermore, our work shows that *HvVP1* and *HvGAMYB* interact in vivo both in yeast (Y2H) and in plant nuclei, in support of the idea that a direct *HvVP1*-*HvGAMYB* protein-protein interaction takes place. Thus, we considered whether the observed negative effects on the *HvGAMYB*-mediated transactivation are derived from the *HvVP1*-*HvGAMYB* interaction. Interestingly, our results show that *HvVP1* interferes with the *HvGAMYB*-BPBF interaction (Y3H assays; Fig. 7,



**Figure 8.** Transactivation assays using, as effectors, the TFs *HvVP1* and BPBF and, as reporter, the *Hor2* gene promoter driving the expression of the *uidA* gene (GUS activity). A, Schematic representation of the effector and reporter constructs used in the analyses. B, Cobombardment experiments on barley developing endosperms using the indicated combinations of effector and reporter constructs. The relative amount of reporter-to-effector plasmids used in these assays corresponds to a 1:1 ratio. Values are means  $\pm$  SE of three independent replicates.



**Figure 9.** Proposed model of the transcriptional regulation of SSP (*Hor2*) and hydrolase (*Amy6.4*) genes in barley seeds mediated by HvVP1 interacting with GAMYB, DOF (BPBF and SAD), BLZ2, and MR1 (MYBR1) TFs during the maturation and postgermination phases. The starchy endosperm and the embryo-plus-aleurone layer are considered separately. Relative gene expression levels are indicated on the y axis. The dotted vertical lines indicate the time of root emergence.



A and B) and also reduces the binding affinity of both HvGAMYB and BPBF for their corresponding cis-motifs in the *HvHor2* promoter (Fig. 7C). However, as reflected in our proposed model (Fig. 9), this repressing activity of HvVP1 is not displayed on maturation genes (e.g. *Hor2*) in the developing starchy endosperm, due to a nonoverlapping temporal pattern of expression. Notably, *HvVP1* transcript levels decrease as maturation progresses while the expression of *HvGAMYB* and *BPBF* increases, hindering the possibility of HvGAMYB being kidnapped by HvVP1 and rendering a fully operative activation complex.

Besides the *HvVP1* expression pattern in the starchy endosperm, its persistence in the developing aleurone and immature embryos would favor the repression of the  $\alpha$ -amylase and other germination-related genes necessary for the acquisition of primary dormancy and for the avoidance of preharvest sprouting, as has been documented previously for its orthologs in maize and Arabidopsis (Parcy et al., 1994; Hoecker et al., 1995).

#### HvVP1 Effects during Barley Germination and Postgermination Phases

After root protrusion (postgermination), different genes encoding hydrolytic enzymes, such as  $\alpha$ -amylases and proteases that mobilize storage reserves, are expressed in the aleurone. In the promoters of these genes, a conserved tripartite cis-acting element, the GA-responsive complex, has been described to contain a GA-responsive element (5'-TAACAAA-3'), the pyrimidine box (5'-CCTTTT-3'), and the 5'-TATCCAC-3' box (Sun and Gubler, 2004). In barley, the interaction of these three cis-elements with TFs belonging to the

MYB-R2R3 (HvGAMYB), DOF (BPBF and three others), and MYBR1-SHAQKYF (HvMCB1 and HvMYBS3) families has been demonstrated (Gubler, et al., 1999; Mena et al., 2002; Isabel-LaMoneda et al., 2003; Rubio-Somoza et al., 2006a, 2006b; Moreno-Risueno et al., 2007). HvGAMYB is considered the master regulator of hydrolase gene expression and is highly induced by GA in the aleurone of germinating seeds (Gubler et al., 1999, 2002). In agreement with our observations during seed maturation, our results show that HvVP1 also interferes with the HvGAMYB transactivation of the *Amy6.4* promoter in germinating aleurone cells (Fig. 4C). Again, this effect is sustained by our disclosure that the interaction with HvVP1 leads to a decreased affinity of GAMYB and other TFs (SAD [HvDOF23]) for their DNA targets as well as to the interference in their protein complex formation. Accordingly, the HvGAMYB-dependent activation of postgermination genes can be achieved only once *HvVP1* expression disappears from the germinating aleurone.

As reflected in our model (Fig. 9), our results show that, upon seed imbibition, *HvVP1* is highly expressed in the mature embryo and in the aleurone up to 24 hoi (the time at which root protrusion occurs) and then decays sharply, in contrast to *HvGAMYB*, whose expression rises up to 48 hoi (Diaz et al., 2002). Taken together, these data indicate that HvVP1 not only prevents the premature expression of postgermination genes (encoding  $\alpha$ -amylases, proteases, etc.) but also that its disappearance during postgermination is required for the mobilization of reserves to take place. The transcriptional repression of hydrolase genes mediated by VP1/ABI3 during germination has been reported previously in monocot and dicot seeds, in species like maize, tomato (*Solanum lycopersicum*), and

Arabidopsis (Hoecker et al., 1999; Nambara et al., 2000; Bassel et al., 2006).

In conclusion, the precise spatiotemporal expression of *HvVP1* allows fine-tuning of the regulation of the maturation and germination programs both within distinct seed compartments and at different developmental stages. As disclosed here, *HvVP1* action occurs mainly by modulating the DNA-binding capacity and protein-protein interactions of TFs, like *HvGAMYB* and *BPDF*, that participate in different regulatory complexes.

The *HvVP1* effects described here rely mostly on its repressing activity or the alternative activation derived from its release. In addition, an intrinsic activation capacity also has been reported for the *VP1/ABI3* proteins. In particular, in nonendosperm seeds, commonly found in dicot plants like *Arabidopsis*, *ABI3* has been described as an activator of maturation (*SSP*) and *LEA* genes, as reflected by their impaired expression in *abi3* mutants (Parcy et al., 1994). Moreover, the ectopic overexpression of *ABI3* (*35S::ABI3*) in conjunction with *bZIP* factors (*AtbZIP10*, *AtbZIP25*, and *AtbZIP53*) induces some of the *SSP* genes in vegetative tissues, in support of the idea that *ABI3* is a positive regulator of *SSP* gene expression (Lara et al., 2003; Alonso et al., 2009). In this respect, the observation that *HvVP1* functions as a transcriptional repressor of the *HvHor2* promoter upon seed maturation is not contradictory, since, in *Arabidopsis*, *SSP* and *LEA* proteins are expressed in the embryo, in contrast to their accumulation in the cereal endosperm. Moreover, globulin storage proteins similar to those of nonendosperm seeds are accumulated in the embryos of cereal seeds and display a similar positive regulation under *VP1*, in support of a conserved function of *VP1/ABI3* in the control of *SSP* gene expression. Hence, the molecular mechanisms underlying the regulatory properties of *VP1/ABI3* proteins seem to be preserved but displayed differentially in the context of endosperm and embryo, where interactions with a different set of transcription factors occur.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Barley (*Hordeum vulgare* 'Bomi') seeds were germinated in the dark, stratified at 4°C for 4 d, and then grown in the greenhouse. Developing endosperms (5, 10, 15, and 20 dap) and germinating seeds (16, 24, 48, and 72 hoi) were frozen in liquid N<sub>2</sub> and stored at -80°C until used for RNA extraction.

Developing endosperms from cv Bomi (15 dap) and aleurone layers peeled off from cv Himalaya germinating seeds (48 hoi) were collected for particle bombardment experiments and used immediately.

### Screening of a Barley Genomic Library

A Lambda phage FIX II genomic library from 8-d-old barley seedlings (cv Igri; Agilent Technologies), representing  $16 \times 10^8$  plaque-forming units mL<sup>-1</sup>, was plated after infection with the *Escherichia coli* strain XL1-Blue MRA (Agilent Technologies). The plaques were transferred onto nylon membranes that were hybridized with a specific probe of a 465-bp fragment obtained by PCR with primers derived from conserved sequences of previously described *VP1* genes

in other cereals (GenBank accession numbers as follows: *Avena fatua*, AJ001140; rice [*Oryza sativa japonica*], D16640.1; *Sorghum bicolor*, AF249881.1; wheat [*Triticum aestivum*], AB047554.1; and maize [*Zea mays*], M60214.1; for primers, see Supplemental Table S2). This fragment spanned the B3 domain and a 3' end conserved region and was  $\alpha$ -<sup>32</sup>P labeled following standard procedures. Pre-hybridization, hybridization, and membrane washing were done as described (Vicente-Carbajosa et al., 1998). The in vivo excision properties of the Lambda phage FIX II vector system allowed the recovery of selected clones in the pBluescript SK plasmid for sequencing (Agilent Technologies).

The *HvVP1* ORF was obtained by PCR from cDNA isolated from developing barley seeds (20 dap; Supplemental Table S2); its corresponding deduced protein appears in Supplemental Figure S1.

### Bioinformatic Resources: *HvVP1* Identification, Phylogenetic Dendrogram, and Transcriptomic Analysis

Identification of the *HvVP1* genomic sequence (AJ431703.1) and its corresponding deduced protein (CAD24413.1) was done using the bioinformatic tools at the European Molecular Biology Laboratory and the National Center for Biotechnology Information databases. The Interpro Program (Pfam database; Bateman et al., 2002; <http://pfam.sanger.ac.uk>) was used to confirm the presence of the B3 DNA-binding domain.

The deduced protein sequences of *VP1* genes were obtained from public databases: those of cv Morex (Mayer et al., 2012); those of cv Himalaya and cv Haruna Nijo (*HvVP1*), wheat (*TaVP1*), *Triticum turgidum* (*TtVP1*), and *Triticum monococcum* (*TmVP1*) from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>); and those of *Brachypodium distachyon* (*BdVP1*), rice (*OsVP1*), and *Arabidopsis* (*AtABI3*) from Phytozome version 8.0 (Goodstein et al., 2012; [www.phytozome.net](http://www.phytozome.net)). These sequences were aligned by means of the ClustalW program (Thompson et al., 1994) and utilized to construct a phylogenetic dendrogram using the neighbor-joining algorithm, a bootstrap analysis with 1,000 replicates, complete deletion, and the Jones-Taylor-Thornton matrix as settings. The MEME program version 6.0 (Tamara et al., 2013) was used to identify conserved motifs within the deduced *VP1* proteins and to validate the phylogenetic tree (Table I). Default parameters were used, except that the maximum number of motifs to find was set to 21 and the minimum width was set to six amino acid residues (Bailey et al., 2009; <http://meme-suite.org/>). A single uppercase letter is given when the frequency of a residue is greater than twice that of the second most frequent one at the same position. A pair of uppercase letters in brackets represents two residues with a relative frequency sum of greater than 75%. When these criteria are not satisfied, a lowercase letter is set when the relative frequency of a residue is greater than 40%; if not, X is given.

The major biochemical parameters of the deduced *VP1* proteins are listed in Supplemental Table S1. Both pI and molecular weight were predicted using the Compute pI/MW tool (Gasteiger et al., 2005; [http://www.expasy.ch/tools/pi\\_tool.html](http://www.expasy.ch/tools/pi_tool.html)).

The in silico transcriptomic analysis of genes *HvVP1*, *HvGAMYB*, *BPDF*, and *BLZ2* in cv Morex (Supplemental Fig. S4) was done using the Hordeum eFP browser at the Bio-Analytic Resource for Plant Biology (<http://bar.utoronto.ca/efpbarley/cgi-bin/efpWeb.cgi>; Druka et al., 2006). For identification of the probe sets for *HvVP1*, *HvGAMYB*, *BPDF*, and *BLZ2* transcription analysis, the PLEXdb BLAST was used ([http://www.plexdb.org/modules/tools/plexdb\\_blast.php](http://www.plexdb.org/modules/tools/plexdb_blast.php)).

### Total RNA Isolation and Northern-Blot Analysis

Total RNA was isolated from 1 to 2 g of barley developing deembryonated seeds (endosperms at 5, 10, 15, and 20 dap), immature (20 dap) and mature dry embryos, germinating aleurones, and germinating embryos (8, 16, 24, 48, and 72 hoi). After electrophoresis and transfer to nylon membranes, hybridization was carried out at 68°C, following standard procedures (Moreno-Risueno et al., 2007). A 280-bp fragment at the 3' untranslated region (nucleotides 3,528–3,808) was used as a specific *HvVP1* probe (Supplemental Table S2). The 18S RNA gene was used as a control for sample charge.

### mRNA in Situ Hybridization Experiments

The protocol described here is a modification of that described by Lara et al. (2003). Barley developing (20 dap) and germinating (24 hoi) seeds were collected and fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, sectioned to 8  $\mu$ m, and dewaxed. An antisense or sense digoxigenin-labeled RNA probe,

corresponding to the same DNA fragment (280 bp) used for northern blots (Supplemental Table S2), was synthesized with the digoxigenin RNA labeling mix, and hybridization was at 56°C overnight. Antibody incubation and color detection were done according to the manufacturer's instructions (Boehringer Ingelheim).

## Yeast One-Hybrid, Y2H, and Y3H Assays

For Y2H assays, the effector plasmids pGBT9 and pGAD424 (Clontech), which contain the alcohol dehydrogenase I (*AdhI*) promoter fused to the *GAL4* DNA-binding domain (GAL4BD; pGBT9 bait vector) and the *GAL4* DNA activation domain (GAL4AD; pGAD424 prey vector), respectively, were used to generate translational fusions with *HvVP1*, *HvGAMYB*, and *BPBF* ORFs or with selected fragments derived from *HvVP1* that were cloned into the *EcoRI*-*BamHI* sites by a PCR strategy. The haploid strains SFY526 and HF7c of *Saccharomyces cerevisiae* (Clontech), carrying the *LacZ* ( $\beta$ -Gal) and *HIS3* (imidazole glycerol phosphate dehydratase) reporter genes, under the control of a truncated *Gal1* promoter that contains *GAL4*-responsive elements (*Gal1UAS*), were used.

For Y3H assays, the *BPBF* and *HvVP1* ORFs were cloned into the pBridge vector (Clontech) in Multiple Cloning Sites I and II, respectively. The *BPBF* ORF was cloned into the *EcoRI*-*BamHI* sites of Multiple Cloning Site I fused to the *GAL4* binding domain, and that of *HvVP1* was cloned into the *NotI*-*BglIII* sites (Multiple Cloning Site II) by a PCR strategy (for primer sequences, see Supplemental Table S2). *GAL4AD*-*HvGAMYB* (pGAD424 vector), used previously for Y2H assays, was used as the third component. The pBridge vector allows the conditional expression of a second gene under the control of the *Met-25* promoter ( $P_{Met-25}$ ) in response to Met levels in the medium.

All Y2H and Y3H assays were done following the manufacturer's instructions (Clontech).

## Bimolecular Fluorescence Complementation

Essentially, *HvVP1* and *HvGAMYB* ORFs were translationally fused to the N- or C-terminal encoding fragment of the *GFP* gene ORF using a PCR strategy and subsequent subcloning into the two versions of the *psmRS-GFP* plasmid (*psmRS-N-GFP* and *psmRS-C-GFP*). The final constructs were used to cobombard inner epidermal cell layers of fresh onion (*Allium cepa*) using a biolistic helium gun device (DuPont PDS-1000; Bio-Rad Laboratories), as described previously (Diaz et al., 2005). The fluorescence emission was observed after 36 h of incubation at 22°C in the dark with a Carl Zeiss Axiophot fluorescence microscope (filter parameters, excitation at 450–490 nm and emission at 520 nm). As a control for nuclei localization, the onion cell layers were stained with 4',6'-diamidino-2-phenylindole (Serva).

## Transient Expression Assays in Barley Developing Endosperms and in Germinating Aleurone Layers

The reporter vectors containing the promoters of genes *Hor2* (encoding a B-hordein) and *Amy6.4* (encoding a high-pI barley  $\alpha$ -amylase),  $P_{Hor2}$  and  $P_{Amy6.4}$  fused to the GUS reporter gene were described previously (Mena et al., 1998, 2002). The effector constructs corresponding to *HvVP1*, *HvGAMYB*, and *BPBF* were prepared by cloning the corresponding ORFs in the pMF6 plasmid under the control of the 35S cauliflower mosaic virus promoter followed by the first intron of the maize *Adh1* gene (35S-I) using a PCR strategy, followed by subcloning at the *EcoRI*-*BamHI* sites of this plasmid. Particle bombardment and subsequent GUS evaluation were carried out with a biolistic helium gun device (DuPont PSD-1000) as described previously (Mena et al., 1998, 2002; Diaz et al., 2002).

## EMSAs

Translational fusions of *HvVP1*, *HvGAMYB*, *BPBF*, *SAD*, and *BLZ2* ORFs to the glutathione S-transferase gene were prepared by cloning their ORFs into the expression vector pGEX-2T (Pharmacia). For this purpose, a PCR strategy was used followed by the subcloning of these ORFs into the *BamHI*-*EcoRI* sites of the pGEX-2T plasmid (Supplemental Table S2). Induction of recombinant proteins with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside and preparation of the *E. coli* protein extracts were performed as described previously (Vicente-Carbajosa et al., 1997).

Two probes, one containing the binding sites for *HvVP1* besides those for *BLZ2* and *BPBF* (PB/GLM; Fig. 7C) and the other containing that for *GAMYB*

(5'-TAACAAC-3') within the *Hor2* promoter ( $P_{Hor2}$ ), were produced by annealing complementary single-stranded oligonucleotides that generate protruding ends (Supplemental Table S2). These probes were end labeled with [<sup>32</sup>P] dATP by the fill-in reaction (Klenow *exo*-free DNA polymerase; United States Biochemical) and purified from 8% PAGE (39:1 cross linking). The DNA-protein binding reactions and the EMSA experiments were done, as described previously, using 1 ng of <sup>32</sup>P-labeled probes (Moreno-Risueno et al., 2008).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Genomic, cDNA, and deduced amino-acid sequences of *HvVP1* cv. Igri.

**Supplemental Figure S2.** Gene copy number of *HvVP1*.

**Supplemental Figure S3.** Comparison of the *HvVP1* deduced sequences.

**Supplemental Figure S4.** Pictographic representation of *HvVP1*, *GAMYB*, *BPBF*, and *BLZ2* transcript accumulation during seed development.

**Supplemental Figure S5.** Transactivation assays using as effectors *HvVP1* and *BPBF*, and as reporter the *Amy6.4* gene promoter.

**Supplemental Figure S6.** Electrophoretic Mobility Shift Assays (EMSA) of the recombinant proteins *BLZ2* (bZIP family) and *SAD* (DOF family) with the PB/GLM probe derived.

**Supplemental Table S1.** Major characteristics of *VP1*/*ABI3* predicted proteins.

**Supplemental Table S2.** Oligonucleotide sequences of primers used.

## ACKNOWLEDGMENTS

We thank Mar González for excellent technical assistance.

Received January 22, 2016; accepted February 4, 2016; published February 8, 2016.

## LITERATURE CITED

- Alonso R, Oñate-Sánchez L, Weltmeier F, Ehlert A, Diaz I, Dietrich K, Vicente-Carbajosa J, Dröge-Laser W (2009) A pivotal role of the basic leucine zipper transcription factor bZIP53 in the regulation of *Arabidopsis* seed maturation gene expression based on heterodimerization and protein complex formation. *Plant Cell* 21: 1747–1761
- Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clemente L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 37: W202–W208
- Bassel GW, Mullen RT, Bewley JD (2006) *ABI3* expression ceases following, but not during, germination of tomato and *Arabidopsis* seeds. *J Exp Bot* 57: 1291–1297
- Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL (2002) The Pfam protein families database. *Nucleic Acids Res* 30: 276–280
- Bewley JD (1997) Seed germination and dormancy. *Plant Cell* 9: 1055–1066
- Braybrook SA, Harada JJ (2008) LECs go crazy in embryo development. *Trends Plant Sci* 13: 624–630
- Casaretto J, Ho TH (2003) The transcription factors *HvABI5* and *HvVP1* are required for the abscisic acid induction of gene expression in barley aleurone cells. *Plant Cell* 15: 271–284
- Diaz I, Martinez M, Isabel-LaMoneda I, Rubio-Somoza I, Carbonero P (2005) The DOF protein, *SAD*, interacts with *GAMYB* in plant nuclei and activates transcription of endosperm-specific genes during barley seed development. *Plant J* 42: 652–662
- Diaz I, Vicente-Carbajosa J, Abraham Z, Martínez M, Isabel-La Moneda I, Carbonero P (2002) The *GAMYB* protein from barley interacts with the DOF transcription factor *BPBF* and activates endosperm-specific genes during seed development. *Plant J* 29: 453–464
- Druka A, Muehlbauer G, Druka I, Caldo R, Baumann U, Rostoks N, Schreiber A, Wise R, Close T, Kleinhofs A, et al (2006) An atlas of gene

- expression from seed to seed through barley development. *Funct Integr Genomics* 6: 202–211
- Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* 171: 501–523
- Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* 59: 387–415
- Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In JK Walker, ed, *The Proteomics Protocols Handbook*. Humana Press, New York, pp 571–607
- González-Calle V, Barrero-Sicilia C, Carbonero P, Iglesias-Fernández R (2015) Mannans and endo- $\beta$ -mannanases (MAN) in *Brachypodium distachyon*: expression profiling and possible role of the BdMAN genes during coleorhiza-limited seed germination. *J Exp Bot* 66: 3753–3764
- González-Calle V, Iglesias-Fernández R, Carbonero P, Barrero-Sicilia C (2014) The BdGAMYB protein from *Brachypodium distachyon* interacts with BdDOF24 and regulates transcription of the *BdCathB* gene upon seed germination. *Planta* 240: 539–552
- Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, et al (2012) Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res* 40: D1178–D1186
- Graeber K, Linkies A, Müller K, Wunchova A, Rott A, Leubner-Metzger G (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae DOG1 genes. *Plant Mol Biol* 73: 67–87
- Gubler F, Chandler PM, White RG, Llewellyn DJ, Jacobsen JV (2002) Gibberellin signaling in barley aleurone cells: control of SLN1 and GAMYB expression. *Plant Physiol* 129: 191–200
- Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pI  $\alpha$ -amylase gene promoter. *Plant Cell* 7: 1879–1891
- Gubler F, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr Opin Plant Biol* 8: 183–187
- Gubler F, Raventos D, Keys M, Watts R, Mundy J, Jacobsen JV (1999) Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. *Plant J* 17: 1–9
- Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK (1992) The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes Dev* 6: 609–618
- Hill A, Nantel A, Rock CD, Quatrano RS (1996) A conserved domain of the *viviparous-1* gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. *J Biol Chem* 271: 3366–3374
- Hobo T, Kowayama Y, Hattori T (1999) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. *Proc Natl Acad Sci USA* 96: 15348–15353
- Hoecker U, Vasil IK, McCarty DR (1995) Integrated control of seed maturation and germination programs by activator and repressor functions of *Viviparous-1* of maize. *Genes Dev* 9: 2459–2469
- Hoecker U, Vasil IK, McCarty DR (1999) Signaling from the embryo conditions Vp1-mediated repression of *alpha*-amylase genes in the aleurone of developing maize seeds. *Plant J* 19: 371–377
- Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytol* 179: 33–54
- Iglesias-Fernández R, Rodríguez-Gacio MC, Barrero-Sicilia C, Carbonero P, Matilla A (2011a) Three endo- $\beta$ -mannanase genes expressed in the micropylar endosperm and in the radicle influence germination of *Arabidopsis thaliana* seeds. *Planta* 233: 25–36
- Iglesias-Fernández R, Rodríguez-Gacio MC, Matilla AJ (2011b) Progress in research on afterripening. *Seed Sci Res* 21: 69–80
- Iglesias-Fernández R, Rodríguez-Gacio MdelC, Barrero-Sicilia C, Carbonero P, Matilla AJ (2011c) Molecular analysis of endo- $\beta$ -mannanase genes upon seed imbibition suggest a cross-talk between radicle and micropylar endosperm during germination of *Arabidopsis thaliana*. *Plant Signal Behav* 6: 80–82
- Iglesias-Fernández R, Wozny D, Iriondo-de Hond M, Oñate-Sánchez L, Carbonero P, Barrero-Sicilia C (2014) The *AtCathB3* gene, encoding a cathepsin B-like protease, is expressed during germination of *Arabidopsis thaliana* and transcriptionally repressed by the basic leucine zipper protein GBF1. *J Exp Bot* 65: 2009–2021
- Isabel-LaMoneda I, Diaz I, Martinez M, Mena M, Carbonero P (2003) SAD: a new DOF protein from barley that activates transcription of a *cathepsin B-like thiol protease* gene in the aleurone of germinating seeds. *Plant J* 33: 329–340
- Jones HD, Peters NC, Holdsworth MJ (1997) Genotype and environment interact to control dormancy and differential expression of the *VIVIPAROUS 1* homologue in embryos of *Avena fatua*. *Plant J* 12: 911–920
- Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. *Curr Opin Plant Biol* 5: 33–36
- Lara P, Oñate-Sánchez L, Abraham Z, Ferrándiz C, Díaz I, Carbonero P, Vicente-Carbajosa J (2003) Synergistic activation of seed storage protein gene expression in Arabidopsis by ABI3 and two bZIPs related to OPAQUE2. *J Biol Chem* 278: 21003–21011
- Marella HH, Quatrano RS (2007) The B2 domain of VIVIPAROUS1 is bi-functional and regulates nuclear localization and transactivation. *Planta* 225: 863–872
- Matsumoto T, Tanaka T, Sakai H, Amano N, Kanamori H, Kurita K, Kikuta A, Kamiya K, Yamamoto M, Ikawa H, et al (2011) Comprehensive sequence analysis of 24,783 barley full-length cDNAs derived from 12 clone libraries. *Plant Physiol* 156: 20–28
- Mayer KF, Waugh R, Brown JW, Schulman A, Langridge P, Platzer M, Fincher GB, Muehlbauer GJ, Sato K, Close TJ, et al (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491: 711–716
- McCarty DR, Carson CB, Stinard PS, Robertson DS (1989) Molecular analysis of viviparous-1: an abscisic acid-insensitive mutant of maize. *Plant Cell* 1: 523–532
- McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* 66: 895–905
- Mena M, Cejudo FJ, Isabel-Lamonedá I, Carbonero P (2002) A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone. *Plant Physiol* 130: 111–119
- Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P (1998) An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamins-box of a native B-hordein promoter in barley endosperm. *Plant J* 16: 53–62
- Moreno-Risueno MA, Díaz I, Carrillo L, Fuentes R, Carbonero P (2007) The HvDOF19 transcription factor mediates the abscisic acid-dependent repression of hydrolase genes in germinating barley aleurone. *Plant J* 51: 352–365
- Moreno-Risueno MA, González N, Díaz I, Parcy F, Carbonero P, Vicente-Carbajosa J (2008) FUSCA3 from barley unveils a common transcriptional regulation of seed-specific genes between cereals and Arabidopsis. *Plant J* 53: 882–894
- Nakamura S, Toyama T (2001) Isolation of a VP1 homologue from wheat and analysis of its expression in embryos of dormant and non-dormant cultivars. *J Exp Bot* 52: 875–876
- Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S (2000) The role of *ABI3* and *FUS3* loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. *Dev Biol* 220: 412–423
- Nonogaki H, Chen F, Bradford KJ (2007) Mechanisms and genes involved in germination *sensu stricto*. In K Bradford, H Nonogaki, eds, *Seed Development, Dormancy and Germination*. Blackwell Publishing, Oxford, pp 264–304
- Oñate L, Vicente-Carbajosa J, Lara P, Díaz I, Carbonero P (1999) Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif of *B-hordein* promoters in barley endosperm. *J Biol Chem* 274: 9175–9182
- Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during *Arabidopsis* seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *Plant Cell* 6: 1567–1582
- Paz-Ares J, Wienand U, Peterson PA, Saedler H (1986) Molecular cloning of the *c* locus of Zea mays: a locus regulating the anthocyanin pathway. *EMBO J* 5: 829–833
- Pritchard SL, Charlton WL, Baker A, Graham IA (2002) Germination and storage reserve mobilization are regulated independently in Arabidopsis. *Plant J* 31: 639–647

- Reidt W, Wohlfarth T, Ellerström M, Czihal A, Tewes A, Ezcurra I, Rask L, Bäuml H (2000) Gene regulation during late embryogenesis: the RY motif of maturation-specific gene promoters is a direct target of the *FUS3* gene product. *Plant J* **21**: 401–408
- Rodríguez MV, Barrero JM, Corbineau F, Gubler F, Benech-Arnold RL (2015) Dormancy in cereals (not too much, not so little): about the mechanisms behind this trait. *Seed Sci Res* **25**: 99–119
- Rubio-Somoza I, Martinez M, Abraham Z, Diaz I, Carbonero P (2006a) Ternary complex formation between HvMYBS3 and other factors involved in transcriptional control in barley seeds. *Plant J* **47**: 269–281
- Rubio-Somoza I, Martinez M, Diaz I, Carbonero P (2006b) HvMCB1, a R1MYB transcription factor from barley with antagonistic regulatory functions during seed development and germination. *Plant J* **45**: 17–30
- Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. *Annu Rev Plant Biol* **55**: 197–223
- Suzuki M, Kao CY, Cocciolone S, McCarty DR (2001) Maize *VP1* complements *Arabidopsis abi3* and confers a novel ABA/auxin interaction in roots. *Plant J* **28**: 409–418
- Swaminathan K, Peterson K, Jack T (2008) The plant B3 superfamily. *Trends Plant Sci* **13**: 647–655
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**: 2725–2729
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680
- To A, Valon C, Savino G, Guillemot J, Devic M, Giraudat J, Parcy F (2006) A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. *Plant Cell* **18**: 1642–1651
- Vasil V, Marcotte WR Jr, Rosenkrans L, Cocciolone SM, Vasil IK, Quatrano RS, McCarty DR (1995) Overlap of Viviparous1 (VP1) and abscisic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. *Plant Cell* **7**: 1511–1518
- Vicente-Carbajosa J, Carbonero P (2005) Seed maturation: developing an intrusive phase to accomplish a quiescent state. *Int J Dev Biol* **49**: 645–651
- Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ (1997) A maize zinc-finger protein binds the *prolamin* box in *zein* gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proc Natl Acad Sci USA* **94**: 7685–7690
- Vicente-Carbajosa J, Oñate L, Lara P, Diaz I, Carbonero P (1998) Barley BLZ1: a bZIP transcriptional activator that interacts with endosperm-specific gene promoters. *Plant J* **13**: 629–640
- Yan D, Duermeyer L, Leoveanu C, Nambara E (2014) The functions of the endosperm during seed germination. *Plant Cell Physiol* **55**: 1521–1533
- Zeng Y, Raimondi N, Kermode AR (2003) Role of an *ABI3* homologue in dormancy maintenance of yellow-cedar seeds and in the activation of storage protein and *Em* gene promoters. *Plant Mol Biol* **51**: 39–49